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RECOMBINANT ADENOVIRAL VECTORS AND
APPLICATIONS THEREOF

The present invention relates to novel recombinant adenoviruses and the method for preparing them, as well as to their uses as expression and gene transfer vectors for vaccinatory purposes or for therapeutic purposes such as the treatment of cancer.

Adenoviruses are naked viruses which possess a linear double-stranded DNA genome of about 30-40 kbp in size, flanked by short inverted repeat sequences (ITRs).

The genome of the adenovirus is organized in early transcription units (E1 to E4) and a late unit (MLTU) which is composed of five transcript families (L1 to L5) whose expression is separated by the initiation of the replication of the viral DNA.

The early phase begins, two hours after infection, with the transcription and sequential expression of the regions E1A then E4, almost simultaneously E3 and E1B, then E2A and finally E2B. The immediate early region E1A encodes transactivators of other early genes of the adenovirus (E1B, E2, E3 and E4) as well as of cellular genes. Replication of the viral DNA begins eight hours after infection. The late phase, which commences twelve hours after infection, is characterized by abolition of the synthesis of the cellular proteins in favor of the late viral proteins, which enter into the structural composition of the adenoviral particle and participate in the assembly of the virion and its release while modifying the structural integrity of the infected cell.

The adenoviruses are particularly attractive for developing viral vectors due to their characteristics and the amount of knowledge which is available with

regard to their genetic organization and their biology.

Different construction strategies have been considered depending on whether the aim is to obtain a replicating 5 virus, which is able to multiply in the host (human or animal), or a nonreplicating virus which is unable to multiply in the host.

Constructing a nonreplicating vector involves deleting 10 a region which is essential for viral replication. The resulting viruses, which are unable to replicate and consequently to produce infectious particles in cells which are permissible for infection by the corresponding wild-type virus, are produced in modified cell 15 lines which are able to supply the products of the deleted genes in *trans*.

A strategy which is commonly used consists in inserting 20 a heterologous gene into the left-hand part of the genome between the left ITR and the E1 region in place of the promoter and of the coding region of the E1A gene (partial E1 deletion) and, where appropriate, of the E1B gene (total deletion of the E1 region). The viruses from which E1A has been deleted are unable to 25 replicate in cells which do not complement the E1A functions. However, they are able to express substantial quantities of exogenous protein in the infected cells.

30 A large number of human adenoviral vectors (Ad2 and Ad5) in which the E1 region and, where appropriate, the E3 region are deleted have been constructed, mainly for the purpose of human gene therapy. Mutations (E2 region) or additional deletions (E2 or E4 region) have 35 been introduced for the purpose of improving these vectors.

Nonreplicating canine adenoviral vectors in which all

the E1 region has been deleted have also been developed for human gene therapy applications [Klonjkowski et al., Human Gene Therapy, 8, 2103-2115, 1997, deletion of positions 411 to 2898 of Cav2; application 5 WO 95/14101 and US patent 5837531 in the name of Rhone Poulenc Rorer; Kremer et al., J. Virol., 74, 505-512, 2000, deletion of positions 412 to 2497 of Cav2].

These nonreplicating vectors have demonstrated good 10 efficiency in transferring genes into a large number of tissues. However, they suffer from a certain number of disadvantages in particular for transferring genes into actively dividing cells such as tumor cells. In these 15 cells, rapid abolition of the expression of the transferred gene is observed, with this abolition being linked to the loss of the extrachromosomal vector during the course of successive divisions.

Constructing a replicating vector involves the 20 necessity of not eliminating any sequence in the viral genome which is essential for its replication and for the production of infectious viral particles in the host (productive viral cycle). Only a small number of heterologous sequence insertion sites which satisfy 25 these requirements are at present known in adeno-viruses.

Replicating vectors have been obtained by inserting 30 heterologous genes into nonessential regions such as the E3 region and the right-hand part of the genome between the right ITR and the transcriptional regulation sequences of the E4 promoter. Replicating vectors have also been obtained by inserting a heterologous gene into the left-hand part of the genome 35 between the left ITR and the E1 region provided that functional E1 genes are preserved. More precisely, insertion of a heterologous sequence between positions 455 and 917 of the human adenovirus (Ad5),

which inactivates the E1A gene by deleting the promoter and a part of the coding region of E1A, is compensated by inserting a copy of this gene into the vector in an ectopic position (Eloit et al., J. Gen. Virol., 76, 5 1583-1589, 1995).

Replicating vectors have been constructed in this way from human (Eloit et al., see above), bovine (Mittal et al., J. Gen. Virol., 76, 93-102, 1995), ovine (Xu 10 et al., Virology, 230, 62-71, 1997), avian (Michou et al., J. Virol., 73, 1399-1410, 1999; Sheppard et al., Arch. Virol., 143, 915-930, 1998), canine (Cav2; international application WO 98/00166 and US patent 6090393, in the name of Rhone Merieux; 15 international application WO 91/11525 and US patent 5616326 in the name of Glasgow University, Morrison et al., Virology, 293, 26-30, 2002) and porcine (Reddy et al., J. Gen. Virol., 80, 563-570, 1999; Tuboly et al., J. Gen. Virol., 82, 183-190, 2001) adeno- 20 viruses.

These replicating adenoviruses have been mainly developed for vaccinatory applications. In a general manner, they have demonstrated a high level of efficacy 25 in connection with inducing immune responses, both as far as the antibody response and the CTL response are concerned (for a review, see Eloit, Virologie [Virology], 2, 109-120, 1998 and Klonjkowski et al., in "Adenoviruses: basic biology to gene therapy", pp. 163- 30 173, P. Seth, Ed., R.G. Landes Company, Austin Texas, USA).

However, these replicating vectors suffer from some drawbacks:

35 - they pose problems of biosafety linked to the risk of these replicating viruses spreading,
- the substantial quantity of the viral particles which is produced by the infected cells leads to a

powerful immune response being induced against the vector and limits the efficacy of repeat injections, neutralization by the maternal antibodies of the vaccinatory antigen which is released from cells which have been destroyed by the infection decreases the efficacy of these replicating vectors in the young animal.

It is apparent that no recombinant adenovirus which makes it possible to efficiently transduce cells, in particular dividing cells such as tumor cells, without involving risks of dissemination into the environment is currently available.

In choosing type 2 canine adenovirus (Cav2) as an experimental model, the inventors sought to determine whether it was possible to identify new insertion sites which made it possible to obtain replicating recombinant adenoviruses.

Thus, they observed that deleting a small proportion of the beginning of the region located between the end of the left ITR and the beginning of the sequence encoding E1A did not affect the ability of the adenoviruses to replicate their genome and to multiply in a permissive host, the site of this deletion can therefore constitute a novel site for inserting heterologous genes.

In addition, the inventors observed that, surprisingly, other deletions in the same region made it possible to obtain adenoviruses which were able to replicate their genome in a permissive host but which were unable to multiply. These adenoviruses will be designated "pseudoreplicating adenoviruses" below.

In that which follows, the positions of the different regions of the adenoviral genome are defined by

reference to the positions of the corresponding regions (that is to say, containing elements having a similar function) of the genome of the type 2 canine adenovirus in the GenBank J04368 sequence.

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Thus, the region located between the end of the left ITR and the beginning of the sequence encoding E1A corresponds to that located between position 311 and position 499 in the GenBank J04368 genomic sequence of 10 type 2 canine adenovirus.

The present invention relates to a recombinant adenovirus which can be obtained from a replicating adenovirus by deleting all or part of the region of the 15 genome of said replicating adenovirus which corresponds to that located between positions 311 and 499 in the genome of type 2 canine adenovirus (GenBank J04368), with said deletion comprising all or part of the region of the genome of the original replicating adenovirus 20 corresponding to that located between positions 311 and 401 in the genome of type 2 canine adenovirus.

According to a first embodiment of a recombinant adenovirus according to the invention, the deleted portion consists of all or part of the region of the 25 genome of the original replicating adenovirus which corresponds to that located between positions 311 and 319 in the genome of type 2 canine adenovirus; this deletion makes it possible to obtain a replicating recombinant adenovirus which is able to multiply in a 30 host which is permissive to infection with an original wild-type adenovirus (productive viral cycle).

According to a second embodiment of a recombinant adenovirus according to the invention, the deleted portion comprises all or part of the region of the 35 genome of the original replicating adenovirus which corresponds to that located between positions 318 and

401 in the genome of type 2 canine adenovirus; this deletion advantageously makes it possible to obtain pseudoreplicating adenoviruses, that is to say adenoviruses which are able to replicate but unable to
5 produce infectious viral particles and therefore unable to multiply (abortive cycle) in a host which is permissive to infection with the original wild-type adenovirus.

10 Obtaining pseudoreplicating adenoviruses according to the invention involves, in particular, eliminating all or part of the putative encapsidation signals of the 5'-TTA/G-3' Ax, AxI and AxII type (respectively located in positions 341-344, 377-380 and 388-391 in the
15 GenBank J04368 Cav2 sequence).

The portion which is deleted in these pseudoreplicating adenoviruses can additionally comprise:

- all or part of the region of the genome of the original replicating adenovirus corresponding to that located between positions 311 and 319 in the genome of type 2 canine adenovirus; and/or
- all or part of the region of the genome of the original replicating adenovirus corresponding to that located between positions 400 and 439 in the genome of type 2 canine adenovirus; this deletion eliminates, in particular, the TATA box of the E1A promoter (located in position 409 in the GenBank J04368 Cav2 sequence);
25 and/or
- all or part of the region of the genome of the original replicating adenovirus corresponding to that located between positions 438 and 499 in the genome of type 2 canine adenovirus; this deletion eliminates, in particular, the E1A transcription initiation site (located in position 439 in the GenBank J04368 Cav2 sequence).
30
35

In all these cases, the (replicating or pseudo-replicating) recombinant adenoviruses according to the invention retain the left ITR sequences which are essential for replication and for activating transcription (4 repeated GGTCA motifs located between positions 62 and 99 in the Cav2 genome) as well as the 5' TTGN₈CG-3' type A_I and 5'-TTTA/G-3' type A_{II} to A_{IX} encapsidation signals (respectively located at positions 197-200, 206-209, 213-216, 226-232, 239-242, 250-253, 258-261, 272-275 and 306-309 in the Cav2 genome). They also retain all of the E1A coding sequence as well as regions of the E1 gene located downstream thereof (E1A polyadenylation signal and E1B region).

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According to a preferred embodiment of a recombinant adenovirus according to the invention, it additionally comprises at least one heterologous sequence of interest inserted in its genome.

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In order to construct a recombinant adenovirus in accordance with this embodiment, said heterologous sequence will, in the case of a replicating adenovirus, be inserted into the region of the genome corresponding to that located between positions 311 and 319 in the genome of type 2 canine adenovirus.

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In the case of a pseudoreplicating adenovirus, said heterologous sequence can also be inserted into this region or at any other site in the region of the genome corresponding to that located between positions 311 and 499 in the genome of type 2 canine adenovirus. The insertion into this region can be effected in place of the deleted portion or in the vicinity thereof.

35

A heterologous sequence can also be inserted into any one of the sites which are normally used for this purpose for constructing replicating adenoviruses. The

insertion can, for example, be effected in the E3 region or in the region located between the E4 region and the right ITR, as described in US patent 6090393, or in the 3' portion of the right ITR, as described in 5 US patent 5616326.

A "heterologous sequence" is understood as meaning any sequence other than that contained between positions 311 and 499 in the genome of said wild-type adenovirus.

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The following may be mentioned as nonlimiting examples:

- genes encoding a vaccinal antigen, for example the gag or env genes of the feline immunodeficiency virus (FIV), the S, M or N protein of the feline 15 coronavirus, a canine or feline parvovirus capsid protein, the G glycoprotein of the rabies virus or the *Leptospira sp.* Hap-1 protein, etc.
- corrective genes which can be used in gene therapy, for example that for erythropoietin (Epo), for vascular endothelium growth factor (VEGF), for neurotrophin 3 (NT-3) or for atrial natriuretic factor (ANF), etc.;
- genes which can be used for treating cancer, for example that for IL-2, that for IFN γ , etc.

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Recombinant adenoviruses according to the invention can, in particular, be derived from mammalian adenoviruses and, in particular, canine adenoviruses, in particular type 2 canine adenoviruses.

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The recombinant adenoviruses according to the present invention can be prepared using customary techniques which are per se well known to the skilled person (cf., for example, GRAHAM and PREVEC (*Manipulation of Adeno-virus Vectors, Methods Mol. Biol.*, 7, 109-128, 1991), in particular using techniques comprising: (i) using standard techniques of double homologous recombination to generate recombinant genomes in *E. coli* and

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(ii) transfecting the resulting recombinant genomes into suitable cell lines which enable said genomes to be amplified and encapsidated in infectious viral particles.

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It will be possible, for example, to use homologous recombination techniques in *E. coli*, such as those described by CHARTIER et al., (J. Virol., 70, 7, 4805-4840, 1996) and in US patent 6110735 in the name 10 of TRANSGENE or else those described by CROUZET et al., (Proc. Nat. Acad. Sci. USA, 94, 1414-1419, 1997). These methods are based on an intermolecular homologous recombination between a "recipient" DNA molecule containing the complete genome of an adenovirus and a 15 "donor" DNA molecule comprising a heterologous sequence to be inserted into said genome flanked by sequences which are homologous with those of the region of the adenoviral genome where it is desired to effect the insertion. The recipient molecule is linearized by 20 cleaving at a restriction site which is unique in the genome of the adenovirus and which is located at the insertion site. Selection of the recombinant genomes is then based on circularization of the recipient molecule.

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These methods therefore suffer from the drawback of only being able to insert said heterologous sequence into a region comprising a restriction site which is unique in the genome of the adenovirus.

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The inventors have now developed a method for inserting a heterologous sequence into an adenovirus, which method does not require linearizing the genome of the adenovirus by cleaving at the insertion site.

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This method differs from that described by CHARTIER et al. in that:

- 1) the heterologous DNA fragment (donor molecule) to

be inserted into the genome of the adenovirus (recipient molecule) comprises a selection marker which makes it possible to isolate the recombinant plasmids on the basis of their double resistance to both ampicillin and kanamycin, and

5 2) said fragment is cotransformed with a recipient molecule which is either in circular form or in a form which has been linearized by cleaving at a restriction site which is located outside the

10 insertion site.

Consequently, the present invention also relates to a method for preparing a recombinant adenovirus by means of intermolecular homologous recombination in a

15 prokaryotic cell, characterized in that it comprises the following steps:

20 α) introducing, into said prokaryotic cell: (i) a plasmid comprising the genome of an adenovirus and a first selection gene; and (ii) a previously linearized DNA fragment which comprises a heterologous sequence to be inserted into said genome flanked by sequences which are homologous to those flanking the site of said plasmid where the insertion is to be effected and which includes a second selection gene which differs from the first; and

25 β) culturing said prokaryotic cell under selective conditions in order to make it possible to generate and select cells which harbor recombinant plasmids which are expressing the first and second selection genes, and

30 γ) isolating the genome of said recombinant adenovirus from selected cells.

35 "Selective conditions" are understood as meaning culture conditions under which the first and second selection agents (for example antibiotics) are present at concentrations which do not allow untransformed

cells to multiply but which allow cotransformed cells to multiply.

According to a first embodiment of the invention, the
5 plasmid which is used in α) is in circular form.

According to a second embodiment of the invention, the
plasmid used in step α) has been previously linearized
by cleaving at a restriction site which is located
10 outside the insertion site.

According to another advantageous embodiment of the
invention, the first and/or second selection gene is a
gene for resistance to an antibiotic, for example a
15 gene for resistance to ampicillin or kanamycin.

According to another embodiment of the invention, the
second selection gene is flanked by 2 identical or
different restriction sites which are absent from the
20 genome of the adenovirus used in step α); this
selection gene can therefore be excised from the
sequence of the genome of the recombinant adenovirus by
digesting at these sites.

25 Advantageously, the method according to the invention
comprises, after preparing the recombinant genome as
described in steps α) to γ) above, an additional step
of transfecting the recombinant genome into a suitable
cell line which enables said genome to be amplified and
30 encapsidated in infectious viral particles.

In order to prepare recombinant adenoviruses according
to the invention, it is possible to use cell lines
which are known per se to the skilled person (cf., for
35 example, GRAHAM and PREVEC, see above) and which are
expressing the E1 region of the adenovirus and, where
appropriate, the E4 region of the adenovirus when this
latter has been impaired by inserting a heterologous

sequence of interest. Cell lines which can be used, and which may be mentioned, in particular, are human cell lines such as the 293 cell line (GRAHAM et al., J. Gen. Virol., 36, 59-74, 1977) and canine cell lines such as 5 the DK/E1-28 cell line (KLONJKOWSKI et al., Human Gene Therapy, see above). Advantageously, it will be possible to use a new cell line which has been constructed by the inventors and which is modified by inserting a fragment consisting of the sequence corresponding to 10 that which extends from position 439 to position 3595 in the genome of the (GenBank J04368) type 2 canine adenovirus; this cell line does not contain the sequences which are located upstream of position 439 and which are present in the above-mentioned cell lines 15 of the prior art.

Said cell line is preferably of canine origin.

20 The invention additionally relates to plasmids and nucleic acid molecules which can be used for preparing the genome of a recombinant adenovirus according to the invention, in particular a canine adenovirus, and to said recombinant genomes which can be obtained by the methods as defined above.

25 The invention relates, in particular, to the following nucleic acid molecules and plasmids:

- any nucleic acid molecule which is selected from the group consisting of:
 - a) a nucleic acid molecule which represents the genome of a recombinant adenovirus according to the invention as defined above;
 - b) a nucleic acid molecule which consists of a fragment of the molecule a) above and which comprises between 10 and 1000 bp, preferably at least 300 bp, of the sequence of the original replicating adenovirus located upstream of the deleted portion and between 10 and 5000 bp,

preferably between 10 and 1000 bp, preferably at least 300 bp, of the sequence of the original replicating adenovirus located downstream of the deleted portion; such a molecule can additionally comprise all or part of a heterologous sequence which is inserted in place of the deleted portion or in proximity thereto.

5
10 - any nucleic acid vector, in particular any plasmid, which contains an a) or b) nucleic acid molecule as defined above.

The invention also relates to the recombinant adenoviruses according to the invention for use as drugs.

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20 The invention relates, in particular, to the use of the adenoviruses according to the invention for preparing immunogenic or vaccinatory compositions or drugs which are intended for gene therapy or for treating cancer as well as for producing recombinant proteins.

Said drug or said composition is preferably intended to be administered to a wild or domestic carnivore, in particular a cat, dog or fox or else a human.

25
30 The recombinant adenoviruses according to the invention are particularly well suited for therapeutic uses, for example vaccinatory therapeutic uses, in man and animals. This is because, contrary to the nonreplicating recombinant adenoviruses, whose genome is present at low copy numbers per cell and rapidly eliminated from actively dividing cells, the replicating or pseudoreplicating recombinant adenoviruses according to the invention multiply significantly in the nucleus of
35 the transduced cells, making it possible to efficiently transduce both quiescent cells and actively dividing cells such as tumor cells. In addition, the pseudoreplicating adenoviruses according to the invention,

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which do not produce any infectious particle, exhibit a high degree of biosafety and also make it possible to induce a strong immune response in connection with repeat injections.

5

Recombinant adenoviruses according to the invention, for example those derived from the canine adenovirus, have applications for vaccinating and treating cancer in domestic or wild carnivores, in particular cats, 10 dogs and foxes. In addition, due to a host tropism of their own, these canine adenoviruses can be used in human gene therapy for targeting tissues which are different from those which can be transduced by the human vectors, for example cells of the central nervous 15 system.

The present invention will be better understood with the help of the remainder of the description which follows and which refers to nonlimiting examples which 20 illustrate the construction and preparation of a recombinant adenovirus according to the invention and its use for expressing genes of interest, in particular for vaccination.

25 **EXAMPLE 1: constructing the adenoviruses CAV 311-319, CAV 311-439 and CAV 311-401**

1) Constructing the recombinant plasmids

30 The following plasmids were constructed using the standard protocols for preparing, cloning and analyzing DNA such as those described in *Current Protocols in Molecular Biology* (Frederick M. AUSUBEL, 2000, Wiley and Son Inc., Library of Congress, USA)

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a) Plasmid pCav2 containing the complete sequence of the genome of type 2 canine adenovirus (Cav2)

This plasmid is constructed by homologous recombination 5 in *E. coli*, in analogy with the method for preparing human adenoviruses, as described in CHARTIER *et al.*, see above.

The main steps involved in constructing this plasmid 10 are shown in figure 1.

More precisely, the left and right ends of the genome of the Manhattan strain of Cav2, corresponding to the sequences at positions 1 to 1060 (fragment A) and 15 29323-31323 (fragment B) are amplified separately by PCR from the genomic DNA of the Cav2 Manhattan strain (APPEL *et al.*, Am. J. Vet. Res., 34, 543-550, 1973) using the following primers:

20 Fragment A

5' - TTGGCGCGCCCATCATCAATAATACAGGAC-3' (SEQ ID NO: 1)

5' - GCTCTAGACCTGCCAACATTAAACC-3' (SEQ ID NO: 2)

Fragment B

25 5' - TTGGCGCGCCCATCATCAATAATACAGGAC-3' (SEQ ID NO: 1)

5' - GCTCTAGAGGGTGATTATTAACAACGTC-3' (SEQ ID NO: 3)

The resulting fragments A and B are separately cloned 30 into the plasmid pCR2.1 (TA Cloning System, INVITROGEN) in order to give, respectively, the plasmids pCR2.1/left ITR and pCR2.1/right ITR. The plasmid pCR2.1/left ITR is digested with BamHI and XbaI and the 1111 bp fragment which is thus generated is cloned between the BamHI and XbaI sites of the plasmid 35 pPolyII Amp^R (GenBank M18128, LATHE *et al.*, Gene, 57, 193-201, 1987) in order to give the plasmid designated pPolyII/left ITR. The plasmid pCR2.1/right ITR is cleaved with BamHI, treated with Klenow polymerase and

then cleaved with *Xba*I; the 2052 bp fragment which is thus generated is cloned between the *Xba*I and *Pvu*II sites of the plasmid pPolyII/left ITR in order to give the plasmid pPolyII.ITRs.Cav2. This plasmid contains 5 the left and right ends of the genome of the Cav2 Manhattan strain which are cloned in the form of a 3073 bp *Asc*I-*Asc*I fragment comprising an *Xba*I site in position 1066 of said fragment, making it possible to linearize the plasmid at the DNA insertion site.

10

The genomic DNA of the Cav2 Manhattan strain and the pPolyII.ITRs.Cav2 DNA, which is linearized at the *Xba*I site, are cotransformed into the *E. coli* strain BJ5183 *recBC sbcBC* (HANAHAN et al., J. Mol. Biol., 166, 15 557-580, 1983). A 33425 bp recombinant plasmid, designated pCav2, is isolated from the colonies which are resistant to ampicillin.

Plasmid pCav2 contains the complete genome of Cav2 20 (Manhattan strain) cloned in the form of a 31331 bp fragment flanked by two *Asc*I sites which are unique in this plasmid, with these sites being absent from the Cav2 genome (Manhattan and Toronto strains) as well as from that of the ovine adenovirus strain OAV.

25

b) Shuttle plasmids

b₁) pShuttle/311-439/CMVeGFP

30 This 6111 bp plasmid is derived from the plasmid pBluescript KS (STRATAGENE) by inserting Cav2 sequences upstream and downstream of the 312-438 deletion (UpRecSeq 1-311 and DownRecSeq 439-1060) at either end of a cassette for expressing the reporter gene GFP.

35

This plasmid is constructed in accordance with the following steps:

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1) a fragment C, corresponding to the sequence at positions 1 to 311 (UpRecSeq) of Cav2, is amplified by PCR using the primers:

5' -TTGGCGCCCATCATCAATAATACAGGAC-3' (SEQ ID NO: 1)

5 5' -CCGACGTCGACCATAAAGTTGACATTAGCCG-3' (SEQ ID NO: 4).

The PCR amplification product is cloned into the plasmid pCR2.1 in order to give the plasmid pCR2.1/UpRecSeq (1-311).

10

2) a fragment D, corresponding to the sequence at positions 439 to 1060 (DownRecSeq) of Cav2, is amplified by PCR using the primers:

5' -GCTCTAGAGCGAAGATCTCCAACAGCAATACACTCTTG-3'

15

(SEQ ID NO: 5)

5' -GCTCTAGACCTGCCAACATTTAAC-3' (SEQ ID NO: 2).

20

The PCR amplification product is cloned into the plasmid pCR2.1 in order to give the plasmid pCR2.1/DownRecSeq (439-1060).

25

3) A fragment E of approximately 2050 bp, containing the early cytomegalovirus promoter, an intron, the eGFP (*enhanced Green Fluorescent Protein*) coding sequence and a polyadenylation signal, is obtained in accordance with the following steps:

30

The plasmid pEGFP-1 (CLONTECH) is cleaved with BamHI, treated with Klenow polymerase and then digested with NotI; the 741 bp fragment which is thus obtained is cloned between the XhoI (previously repaired by treating with the Klenow polymerase) and NotI sites of the plasmid pCI (PROMEGA) in order to give the plasmid pCMVeGFP.

35

The pCMVeGFP plasmid is then cleaved with BglII, treated with Klenow polymerase and then digested with BamHI in order to generate a 2050 bp fragment (fragment E).

4) The fragment E is inserted between the SmaI and BamHI sites of the plasmid pBLUESCRIPT KS in order to give the plasmid pKS/CMVeGFP. The plasmid 5 pCR2.1/UpRecSeq (1-311) is cleaved with KpnI and SalI and the 371 bp fragment thus obtained (fragment C) is cloned between the KpnI and SalI sites of the plasmid pKS/CMVeGFP in order to give the plasmid pKS/CMVeGFP-C. The plasmid 10 pCR2.1/DownRecSeq (439-1060) is cleaved with XbaI and the 650 bp fragment thus obtained (fragment D) is inserted into the XbaI site of the plasmid pKS/CMVeGFP-C in order to give the plasmid designated pShuttle 311-439/CMVeGFP.

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b₂) pShuttle 311-401/CMVeGFP

The shuttle plasmid pShuttle 311-401/CMVeGFP is constructed from the plasmid pShuttle 311-439/CMVeGFP 20 in accordance with the following steps:

The sequence 401-1060 (DownRecSeq) is amplified by PCR using the primers:

5' -GATAAGGATCACGCCGGCTTAAATTCTCAG-3' (SEQ ID NO: 6)

25 5' -GCTCTAGACCTGCCAACATTTAAC-3' (SEQ ID NO: 2).

The PCR amplification product is cloned in the plasmid pCR2.1 in order to give the plasmid pCR2.1/DownRecSeq (401-1060).

30

This plasmid pCR2.1/DownRecSeq (401-1060) is digested with EcoRI and then treated with Klenow polymerase and the 401-1060 fragment thus obtained is substituted for the 439-1060 fragment of the plasmid pShuttle 35 311-439/CMVeGFP, which has been previously digested with XbaI and then treated with Klenow polymerase, in order to give the plasmid pShuttle 311-401/CMVeGFP.

b₃) pShuttle 311-319/CMVeGFP

The shuttle plasmid pShuttle 311-319/CMVeGFP is constructed from the plasmid pShuttle 311-439/CMVeGFP 5 in accordance with the following steps:

The 319-1060 sequence (DownRecSeq) is amplified by PCR using the primers:

5'-GATAAGGATCAACAGAAACACTCTGTTCTCTG-3' (SEQ ID NO: 7)

10 5'-GCTCTAGACCTGCCAACATTAAACC-3' (SEQ ID NO: 2).

The PCR amplification product is cloned into the plasmid pCR2.1 in order to give the plasmid pCR2.1/DownRecSeq (319-1060). 15

This plasmid pCR2.1/DownRecSeq (319-1060) is digested with EcoRI and then treated with Klenow polymerase and the 319-1060 fragment thus obtained is substituted for the 439-1060 fragment of the plasmid pShuttle 20 311-439/CMVeGFP, which has been previously digested with XbaI and then treated with Klenow polymerase, in order to give the plasmid pShuttle 311-319/CMVeGFP.

b₄) Shuttle plasmid pShuttle 311-439/CMVeGFP/Kana

25 This 7027 bp plasmid, which is derived from the plasmid pShuttle 311-439/CMVeGFP by cloning, into the *PmeI* site, a cassette for expressing a gene for resistance to kanamycin in the opposite orientation to that of the 30 cassette for the GFP, is obtained in accordance with the following steps:

The reading frame encoding the Kana gene is amplified by PCR from the plasmid pET-29a(+) (NOVAGEN) using the 35 primers:

5'-AGCTTGTTAACGGCGCGCCGGGATTTGGTCATGAAC-3'

(SEQ ID NO: 8)

5'-CCGGCGCGCCGTTAACAAAGCTATCCGCTCATGAA-3' (SEQ ID NO: 9).

The PCR amplification product is cloned into the plasmid pCR2.1 in order to give the plasmid pCR2.1-Kana/PmeI. The plasmid pCR2.1/Kana/PmeI is cleaved with 5 EcoRI and treated with Klenow polymerase and the fragment of approximately 959 bp in size, containing the reading frame encoding the Kana gene, is inserted into the EcoRV site of the plasmid pShuttle 311-439/CMVeGFP in order to give the plasmid pShuttle 10 311-439/CMVeGFP/Kana.

This plasmid pShuttle 311-439/CMVeGFP/Kana, which is depicted in figure 2 and which contains a gene for resistance to an antibiotic cloned between the 15 adenoviral sequences which are the target of the recombination, upstream of the heterologous sequence to be inserted, advantageously makes it possible to select the recombinants which are resistant to both ampicillin and kanamycin. In addition, the Kana gene, which is 20 then excised from the recombinant plasmid by digesting at the 2 PmeI sites, is absent from the recombinant adenovirus sequence which is generated from this plasmid.

25 b₄) Shuttle plasmid pPoly II 311-439/CMVeGFP/Kana
(figure 2)

This 6332 bp plasmid is obtained by cloning the 4292 bp KpnI-PvuII fragment of pShuttle 311-439/CMVeGFP between 30 the KpnI (position 42) and PvuII (position 63) sites of the plasmid pPoly II, as illustrated in figure 2.

c) **Plasmid pCav 311-439/CMVeGFP**

35 This plasmid is obtained by homologous recombination in the *E. coli* strain BJ5183 in accordance with the following 2 alternatives c1 and c2, which are respectively illustrated by figures 3 and 4:

c₁) Recombination of pShuttle 311-439/CMVeGFP.Kana with plasmid pCav2 in circular form (figure 3)

5 1) the donor molecule, containing the upstream (UpRecSeq 1-311) and downstream (DownRecSeq 439-1060) recombination sequences and the CMVeGFP and Kana cassettes, is prepared from the plasmid pShuttle 311-439/CMVeGFP.Kana by digesting with
10 the restriction enzymes KpnI and EcoRV,

2) the fragment obtained in 1) and the plasmid pCav2 (Amp^R) in circular form are cotransformed into the *E. coli* strain BJ5183, and
15 3) the recombinant plasmids are isolated on the basis of the criterion of resistance to both ampicillin and kanamycin. The sequence of one of them, pCav 311-439/CMVeGFP/Kana, is confirmed by enzymic
20 restriction and by sequencing.

25 4) The Kana cassette is then excised by digesting with the restriction enzyme *PmeI* so as to obtain the plasmid pCav 311-439/CMVeGFP, which contains the Cav2 genome from which the 312-438 sequence has been deleted and replaced with a cassette for expressing GFP.

30 c₂) Recombination of pPoly II 311-439/CMVeGFP.Kana with the plasmid pCav2, which has been previously linearized outside the insertion site (figure 4)

35 1) the donor molecule is prepared from the pPoly II 311-439/CMVeGFP.Kana plasmid by digesting with the restriction enzyme *SwaI*,

2) the plasmid pCav2 is linearized by cleaving at the *PvuI* site,

3) the fragment obtained in 1) and the linearized pCav2 plasmid obtained in 2) are cotransformed into the *E. coli* strain BJ5183, and

5

4) the recombinant plasmids are isolated on the basis of the criterion of resistance to both ampicillin and kanamycin. The sequence of one of them, pCav 311-439/CMVeGFP.Kana, is confirmed by enzymic restriction and by sequencing.

10

5) The Kana cassette is then excised by digesting with the restriction enzyme *PmeI* so as to obtain the plasmid pCav 311-439/CMVeGFP, which contains the Cav2 genome from which the sequence at positions 312-438 has been deleted, with this sequence being replaced by a cassette for expressing GFP.

15

d) **Plasmid pCav 311-401/CMVeGFP**

20 The plasmid pShuttle 311-401/CMVeGFP is digested with *KpnI* and *SwaI* and the 3167 bp fragment thus obtained, and the plasmid pCav 311-439 CMVeGFP which is linearized at the *PmeI* site, are cotransformed into the *E. coli* strain BJ5185. The recombinant plasmid pCav 311-401 CMVeGFP, which is generated by homologous recombination, is selected on the basis of the criterion of resistance to both ampicillin and kanamycin.

25

30 e) **Plasmid pCav 311-319/CMVeGFP**

The plasmid pShuttle 311-319/CMVeGFP is digested with *KpnI* and *SwaI* and the 3249 bp fragment which is thus obtained, and the plasmid pCav 311-439 CMVeGFP which has been linearized at the *PmeI* site, are cotransformed into the *E. coli* strain BJ5185. The recombinant plasmid pCav 311-319 CMVeGFP, which is generated by homologous

35

recombination, is selected on the basis of the criterion of resistance to both ampicillin and kanamycin.

5 2) Producing recombinant viruses

The plasmids pCav 311-439/CMVeGFP, pCav 311-401/CMVeGFP or pCav 311-319/CMVeGFP are digested with the restriction enzyme *AscI* in order to excise the sequences of 10 the recombinant adenovirus genome. The excised adenoviral genome is then transformed into the canine cell line DK/E1-28, which constitutively expresses the Cav2 E1 region (KLONJKOWSKI et al., Human Gene Therapy, see above), in the presence of lipofectamine (GIBCO) in 15 accordance with the customary techniques which are well known per se to the skilled person (cf., for example, GRAHAM and PREVEC, see above). When a cytopathic effect is observed, the virus is harvested from the transfected cells, then amplified in the same DK/E1-28 20 cell line and purified by centrifugation through a cesium chloride gradient using a standard protocol, such as described, for example, in GRAHAM and PREVEC, see above.

25 The genomic sequence of the viruses Cav 311-439/CMVeGFP, Cav 311-401/CMVeGFP and Cav 311-319/CMVeGFP is confirmed by enzymic restriction and by partial sequencing of the viral DNA, which is extracted from the infected DK/E1-28 cells and prepared 30 in accordance with the HIRT technique (J. Mol. Biol., 26, 365-369, 1967). The recombinant Cav virus preparations are titrated by limiting dilution on 96-well plates in accordance with the method of SPEARMAN and KÄRBER (*Virology Methods Manual*, Brian W J Mahy and 35 Hillar O Kangro, 1996, Academic Press, Harcourt Brace & Company). The TCID₅₀/ml titer which is obtained by this method is equivalent to the pfu/ml titer which is obtained by the method plaques on DK cells, in

accordance with the protocol described in KLONJKOWSKI et al., see above.

The following results are obtained:

5

- the isolated viruses Cav 311-439/CMVeGFP, Cav 311-401/CMVeGFP and Cav 311-319/CMVeGFP have a restriction profile and a sequence which is in accordance with those which are expected,
- 10 - the purified viruses Cav 311-439/CMVeGFP, Cav 311-401/CMVeGFP and Cav 311-319/CMVeGFP have a titer of approximately $10^{9.2}$ pfu/ml.

EXAMPLE 2: CHARACTERIZING THE RECOMBINANT VIRUS
15 CAV 311-439

1) Analyzing the efficiency of transduction and the cytopathic effect of Cav 311-439 CMVeGFP in feline and canine cells

20

Canine (DK/E1-28 and DK) or feline (CRFK) cell lines are infected with the virus Cav 311-439 CMVeGFP at a multiplicity of infection of 10 pfu/cell. Uninfected cells and cells which are infected with the wild-type 25 Cav virus (Cav2) are used as controls.

30

At 3 and 5 days after the infection, the presence of a cytopathic effect (EPE) is analyzed by observing infected cells in an optical microscope. In addition, the expression of the transgene by the Cav 311-439 CMVeGFP virus in the infected cells is confirmed by detecting the GFP by fluorescence microscopy.

35

The results of this experiment are presented in tables I and II below:

TABLE I

Virus	DK	DK/E1-28	CRFK
Cav 311-439 CMVeGFP	-	++	-
Cav	+	++	-
Control	-	-	-

TABLE II

5

Cells infected with Cav 311-439.CMVeGFP	ECP	GFP
DK	-	+
DK/E1-28	++	++

These results show that:

- a high level of expression of the transgene by the Cav 311-439 CMVeGFP virus is observed in the infected cells, and
- no cytopathic effect is observed in the unmodified canine cells (DK cells) or feline cells which are infected with this virus Cav 311-439 CMVeGFP; a substantial cytopathic effect is only observed in the canine cells which are infected with this Cav 311-439 CMVeGFP virus and which are expressing the E1 region,
- in the controls, a substantial cytopathic effect is observed in the canine cells (DK and DK/E1-28) which are infected with the wild-type Cav (Cav2) and no cytopathic effect is observed in the feline cells which are infected with Cav2.

The results of these experiments demonstrate that the Cav 311-439 viruses are able to very efficiently transduce the cells without inducing any cytopathic effect in the canine cells which are permissive for replication of the wild-type canine adenoviruses, or in the feline cells.

2) Analyzing the replication of Cav 311-439 CMVeGFP in the feline and canine cells

5 Canine (DK/E1-28 and DK) or feline (CRFK) cell lines are infected with the virus Cav 311-439 CMVeGFP at a multiplicity of infection of 10 pfu/cell. Uninfected cells and cells infected with the wild-type Cav virus are used as controls.

10 At 2, 24, 48 and 72 hours after the infection, the cells are harvested and centrifuged. The intracellular viral DNA is prepared using the HIRT technique (J. Mol. Biol., 26, 365-369, 1967), digested with the enzyme EcoRI and then visualized on an agarose gel following 15 electrophoretic migration.

The results are depicted in figure 5:

20 Legend to figure 5: viral DNA extracted from feline (CRFK) or canine (DK, DK/E1-28) cells at different times after infection (2, 24, 48 and 72 hours) with the adenovirus Cav 311-439.CMVeGFP is digested with EcoRI and analyzed on an agarose gel. The cell line DK/E1-28, which expresses the E1 region of the adenovirus, is 25 used as a positive control for replication.

These results show that:

- the Cav 311-439 CMVeGFP virus replicates its genome in the tested canine and feline cell lines,
- 30 - the level of replication is greater in the canine cells than in the feline cells,
- the peak of replication is reached at 24 hours in the DK/E1-28 cells and at 48 hours in the DK cells, probably because of the cellular expression 35 of the E1 region in the DK/E1-28 cells.

By comparison, in the control cells infected with the wild-type Cav virus, a similar quantity of genomic DNA

is observed in the 3 cell lines.

The results of this experiment demonstrate that the 311-439 deletion does not affect the replication of the
5 canine adenovirus: the vectors which carry this deletion (Cav 311-439 CMVeGFP) behave like the wild-type adenoviruses as far as the replication of their genome in canine or feline cells is concerned.

10 3) Analyzing the production of viral particles in the canine cells infected with the virus Cav 311-439.CMVeGFP

DK canine cell lines are infected with the vector Cav 311-439 CMVeGFP at a multiplicity of infection of,
15 respectively, 0.1, 1 and 10 pfu/cell. Uninfected cells and cells infected with the wild-type Cav virus are used as controls.

20 The infected cells are harvested at 2 hours and 6 days after the infection, and lysed by several cycles of freezing and thawing. The cell lysate is titrated by the abovementioned technique of limiting dilutions.

25 The quantity of virus present in the cells, expressed in pfu/ml, is shown in table III below.

TABLE III

Virus	Time	0.1 pfu/cell	1 pfu/cell	10 pfu/cell
Cav 311-439	D0	< 10 ^{1.8}	10 ^{2.4}	10 ³
	D6	< 10 ^{1.8}	10 ³	10 ^{2.8}
Cav	D0	10 ²	10 ³	10 ^{4.4}
	D6	10 ^{7.6}	10 ^{6.8}	10 ^{6.8}

30 These results show that the Cav 311-439 virus does not produce infectious viral particles in canine cells, such as the DK cells, which are not expressing the E1 region: the Cav 311-439 viral cycle is abortive in the

canine cells.

4) Vaccinating conventional cats with the Cav 311-439 virus

5

Groups of cats are inoculated intramuscularly with the following doses of Cav 311-439:

group 1 (n = 2): $9.6 \cdot 10^7$ pfu

group 2 (n = 2): $9.6 \cdot 10^6$ pfu

10 group 3 (n = 2): control.

On D14, D21 and D31, the serum anti-eGFP antibodies are titrated by ELISA.

15 The results are depicted in figure 6.

Legend to figure 6: serum anti-eGFP antibody (Ab) titers in the cat on days D7, D14, D21 and D31 after the inoculation of different doses of the 20 Cav 311-439.CMVeGFP virus: -■- $9.6 \cdot 10^7$ pfu/ml (pfu: plaque forming units), -▲- $9.6 \cdot 10^7$ pfu/ml, ···□··· $9.6 \cdot 10^6$ pfu/ml, ···○··· $9.6 \cdot 10^6$ pfu/ml.

25 These results show that a single injection of Cav 311-439 induces a (humoral) immune response in the cat which is specific for the gene of interest.

EXAMPLE 3: CONSTRUCTING A CANINE-DERIVED CELL LINE WHICH CONSTITUTIVELY EXPRESSES THE CAV2 E1 REGION

30

A new cell line expressing the E1 region is constructed from the DK cell line (immortalized line of dog kidney cells; ATCC CRL 6247) by means of the following steps:

35 The sequence at positions 439 to 3595 of Cav2 (Manhattan strain) is amplified by PCR using the following primers:

5'-CGGCCGACTCTTGAGTGCGCAGCGAGA-3' (SEQ ID NO: 10)

5'-GGCGCGCCGAGAGACAACGCTGGACACGG-3' (SEQ ID NO: 11).

The PCR amplification product is cloned into the plasmid pCR2.1 to give the plasmid pCR2.1/E1.

5

The plasmid pTRE (CLONTECH) is digested with BamHI, treated with Klenow polymerase and recircularized in order to give the plasmid pTRE/dl BamHI.

10 The plasmid pCR2.1/E1 is digested with EcoRI and the 3187 bp fragment which is thus obtained is cloned into the EcoRI site of the plasmid pTRE/dl BamHI in order to give the plasmid pTRE E1 Cav2.

15 This plasmid pTRE E1 Cav2 contains the coding sequence for the E1A protein under the control of a minimum CMV promoter and response elements of the Tet operon (*Tet-Responsive Element or TRE*), the sequences coding for the E1B proteins (133R and 438R; SHIBATA et al.,
20 Virology, 172, 460-467, 1989) under the control of their own promoter and the endogenous polyadenylation signals for these sequences.

25 Using pTRE E1 Cav2 as the starting material, a cell line expressing the E1 region is obtained in an analogous manner to that used for obtaining the DK/E1-28 cell line (KLONJKOWSKI et al., see above).

30 More precisely, the DK cells are cotransfected with the pTRE E1 Cav2 plasmid, which is linearized at the AatII site, and with the plasmid pTK-Hyg (CLONTECH), which is linearized at the AseI site. Clones are selected in the presence of hygromycin (150 µg/ml) and then analyzed by Southern blotting, Northern blotting, RT-PCR and
35 Western blotting. 4 clones which expressed the E1 region (E1A and E1B), and which were able to efficiently produce the deleted vectors according to the invention, were isolated.

EXAMPLE 4: IMMUNIZING MICE WITH THE CAV 311-319 VIRUS

5 Mice which had been divided into three groups were inoculated intramuscularly with a 10^8 pfu dose of the following viruses:

Group 1 (n = 4): Cav 311-319 eGFP

Group 2 (n = 4): Cav 311-319 CONTROL

Group 3 (n = 1): uninoculated control.

10

The Cav 311-319 CONTROL virus is isogenic with the Cav 311-319 eGFP virus apart from the heterologous gene which is inserted (a heterologous gene encoding a protein which does not have any antigenic relationship with GFP is inserted in place of the gene encoding GFP).

On D7, the serum anti-eGFP antibodies which were produced by the inoculated mice were titrated by Elisa.

20

The results are depicted in figure 7.

Legend to figure 7: OD at 405 nm for different dilutions (from 1 to 8, respectively, 1/5, 1/15, 1/45, 25 1/135, 1/405, 1/1215, 1/3645 and 1/10935) of the mouse sera of the following groups: eGFP (♦), CONTROL (■) and control (▲).

These results show that a single injection of 30 Cav 311-319 induces a (humoral) immune response in the mouse which is specific for the heterologous gene which is inserted in this adenovirus.